BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Problem Image Mailbox.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 September 2001 (07.09.2001)

PCT

(10) International Publication Number
WO 01/64850 A1

(51) International Patent Classification[7]: C12N 5/10, 15/82, 5/04, A01H 4/00

(21) International Application Number: PCT/US01/06271

(22) International Filing Date: 28 February 2001 (28.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/185,658 29 February 2000 (29.02.2000) US

(71) Applicants *(for all designated States except US)*: **AUBURN UNIVERSITY** [US/US]; 309 Samford Hall, Auburn University, AL 36849 (US). **UNIVERSITY OF CENTRAL FLORIDA** [US/US]; 4000 Central Florida Boulevard, Orlando, FL 32816 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **DANIELL, Henry** [US/US]; 1255 Marina Point, #315, Casselberry, FL 32707 (US). **LEE, Seung-Bum** [KR/US]; 12722 Research Parkway, Orlando, FL 32826 (US). **BYUN, Myung, Ok** [KR/US]; 12722 Research Parkway, Orlando, FL 32826 (US).

(74) Agents: **WEISER, Gerard, J.** et al.; 1600 Market Street, Suite 3600, Philadelphia, PA 19103-7286 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with amended claims*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/64850 A1

(54) Title: GENETIC ENGINEERING OF DROUGHT TOLERANCE VIA A PLASTID GENOME

(57) Abstract: This invention provides a method of conferring osmoprotection to plants. Plant plastid genomes, particularly the chloroplast genome, is transformed to express an osmoprotectant. The transgenic plants and their progeny display drought resistance. More importantly, such transgenic plants display no negative pleiotropic effects such as sterility or stunted growth.

GENETIC ENGINEERING OF DROUGHT TOLERANCE VIA A PLASTID GENOME

CROSS-REFERENCES TO RELATED APPLICATIONS

This patent application claims the benefit of U.S. Provisional Application No. 60/185,658, filed 2/29/2000. This earlier provisional application is hereby incorporated by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

The work of this invention is support in part by the USDA-NRICGP grants 95-82770, 97-35504 and 98-0185 to Henry Daniell.

FIELD OF INVENTION

This application pertains to the field of genetic engineering of plant plastid genomes, particularly chloroplasts and to methods of transforming plants to confer or increase drought tolerance and engineered plants which are drought tolerant.

DESCRIPTION OF RELATED ART

Patents of Interest

Londesborough et. al., in U.S. patent no. 5,792,921 (1998), entitled "Increasing the trehalose content of organisms by transforming them with combinations of the structural genes for trehalose synthase," and U.S. patent no. 6,130,368 (2000), entitled "Transgenic plants producing trehalose", proposed a method for increasing trehalose content in various organisms through nuclear transformation.

Hoekema, in U.S. patent no. 5,925,804 (1999), entitled "Production of Trehalose in Plants," proposes a method of engineering plants to produce trehalose. This patent suggests the transformation of plants by introducing to the plant nuclear genome any trehalose phosphate synthase gene driven by an appropriate promoter.

Strom, et al., in U.S. patent no. 6,133,038 entitled "Methods and compositions related to the production of trehalose" (2000), described the genes involved in the biosynthesis of trehalose, trehalose synthase and trehalose-6-phosphate. Methods for producing trehalose biosynthetic enzymes in a host cell through transformation of the cell's nucleus are also proposed. In addition, the patent also suggests nuclear transgenic host cells which contain recomvinant DNA constructs encoding for a trehalose synthase, trehalose phosphatase or both trehalose synthase and, trehalose phosphatase.

BACKGROUND OF THE INVENTION

Effects of increased trehalose accumulation

Water stress due to drought, salinity or freezing is a major limiting factor in plant growth and development. Trehalose is a non-reducing disaccharide of glucose and its synthesis is mediated by the trehalose-6-phosphate (T6P) synthase and trehalose-6-phosphate phosphatase complex in *Saccharomyces cerevisiae*. In *S. cerevisiae*, this complex consists of at least three subunits performing either T6P synthase (TPS1), T6P phosphatase (TPS2) or regulatory activities (TPS3 or TSLI). Trehalose is found in diverse organisms including algae, bacteria, insects, yeast, fungi, animal and plants. Because of its accumulation under various stress conditions such as freezing, heat, salt or drought, there is general consensus that trehalose protects against damages imposed by these stresses. Trehalose is also known to accumulate in anhydrobiotic organisms that survive complete dehydration , the resurrection plant and some desiccation tolerant angiosperms. Trehalose, even when present in low concentrations, stabilizes proteins and membrane structures under stress because of the glass transition temperature, greater flexibility and chemical stability / inertness.

Prior efforts to engineer plants for trehalose production

There have been several efforts to generate various stress resistant transgenic plants by introducing gene(s) responsible for trehalose biosynthesis, regulation or degradation. When trehalose accumulation was increased in transgenic tobacco plants by over-expression of the yeast TPS1, trehalose accumulation resulted in the loss of apical dominance, stunted growth, lancet-shaped leaves and some sterility. Altered phenotype was always correlated with drought tolerance, plants showing severe morphological alterations had the highest tolerance under stress conditions.

Advantages of transforming plants through the chloroplast

In order to minimize the pleiotropic effects observed in the nuclear transgenic plants accumulating trehalose, this invention compartmentalizes trehalose accumulation within chloroplasts. Several toxic compounds expressed in transgenic plants have been compartmentalized in chloroplasts, even through no targeting sequence was provided indicating that this organelle could be used as a repository like the vacuole. Also, osmoprotectants are known to accumulate inside chloroplasts under stress conditions. Inhibition of trehalase activity is known to enhance trehalose accumulation in plants. Therefore, trehalose accumulation in chloroplast may be protected from trehalase activity in the cytosol, if trehalase was absent in the chloroplast.

In addition, chloroplast transformation has several other advantages over nuclear transformation. A common environmental concern about nuclear transgenic plants is the escape of foreign genes through pollen or seed dispersal, thereby creating super weeds or causing genetic pollution among other crops. The latter has resulted in several lawsuits and shrunk the European market for organic produce from Canada from 83 tons in 1994-1995 to 20 tons in 1997-1998. These are serious environmental concerns, especially when plants are genetically engineered for drought tolerance, because of the possibility of creating robust drought tolerant weeds and passing on undesired pleiotropic traits to related crops. Chloroplast transformation should also overcome some of the disadvantages of nuclear transformation that result in lower levels of foreign gene expression, such as gene suppression by positional effect or gene silencing.

Chloroplast genetic engineering has been successfully employed to address aforementioned concerns. For example, chloroplast transgenic plants expressed very high level of insect resistance, due to expression of 10,000 copies of foreign genes per cell, thereby overcoming the problem of insect resistance observed in nuclear transgenic plants. Similarly, chloroplast derived herbicide resistance overcomes out-cross problems of nuclear transgenic plants because of maternal inheritance of plastid genomes. This invention thus presents a solution to the pitfalls of nuclear expression of TPS1 in transgenic plants.

Non-obvious nature of the invention.

Trehalose is a non-reducing disaccharide of glucose and is found in diverse organisms including algae, bacteria, insects, yeast, fungi, animal and plants. Because of its accumulation under various stress conditions such as freezing, heat, salt or drought, there is general consensus that trehalose protects against damages imposed by these stresses. Trehalose is also known to accumulate in anhydrobiotic organisms that survive complete dehydration, the resurrection plant and some desiccation tolerant angiosperms.

There have been several efforts to generate various stress resistant transgenic plants by introducing gene(s) responsible for trehalose biosynthesis, regulation or degradation. When trehalose accumulation was increased in nuclear transgenic tobacco plants by over-expression of the yeast *TPS1*, trehalose accumulation resulted in the loss of apical dominance, stunted growth, lancet shaped leaves and some sterility. Altered phenotype was always correlated with drought tolerance; plants showing severe morphological alterations had the highest tolerance under stress conditions. Prior to this invention, it was not obvious that accumulation of trehalose within plastids would minimize

the pleiotropic effects observed in the nuclear transgenic plants accumulating trehalose or damage plastids. There were no prior reports of trehalose accumulation within plastids or localization of enzymes of trehalose biosynthetic pathway within plastids.

Osmoprotectants are known to accumulate inside chloroplasts under stress conditions but their mode of action is to provide osmotic protection by accumulation of such compounds (as sugars or amino acids) in large quantities. This invention demonstrates that the protection is offered by accumulation of small quantities of trehalose which was not adequate to provide protection from dehydration but rather stability of biological membranes. Inhibition of trehalase activity is known to enhance trehalose accumulation in the cytosol but there are no reports of the presence or absence of trehalase within plastids. Therefore, it was unanticipated that trehalose accumulation within plastids would be protected from trehalase activity. Prior to this invention, there were no reports of using plastid transformation as a strategy to confer drought tolerance to transgenic plants.

BRIEF SUMMARY OF THE INVENTION

This invention provides a method to transform plants through the plastids, particularly chloroplasts, to confer drought tolerance to plants. The vectors with which to accomplish the chloroplast transformation is provided. The transformed plants and their progeny are provided. The transformed plants and their progeny display drought resistance. More importantly, they display no negative pleiotropic effects such as sterility or stunted growth.

The present invention is applicable to all plastids of plants. These include chromoplasts which are present in the fruits, vegetables and flowers; amyloplasts which are present in tubers like the potato; proplastids in roots; leucoplasts and etioplasts, both of which are present in non-green parts of plants.

The present invention provides a method to increase water stress tolerance in dicotyledonous or a monocotyledonous plant, comprising introducing an expression cassette into the cells of a plant to yield transformed plant cells. Plant cells include cells of monocotyledenous plants such as cereals, including corn (Zea mays), wheat, oats, rice, barley, millet and cells of dicotyledenous plant such as soybeans and vegetables like peas. The expression cassette comprises a preselected DNA sequence encoding an enzyme which catalyzes the synthesis of an osmoprotectant, operably linked to a promoter functional in the chloroplast plant cell. The enzyme encoded by the DNA sequence is expressed in the transformed plant cells to increase the level of osmoprotection so as to render the

transformed cells substantially tolerant or resistant to a reduction in water availability that inhibits the growth of untransformed cells of the plant.

As used herein, an "osmoprotectant" is an osmotically active molecule which, when that molecule is present in an effective amount in a cell or plant, confers water stress tolerance or resistance, or salt stress tolerance or resistance, to the cell or plant; when present in lower amounts in a cell or plant, an "osmoprotectant" confers membrane stability. Those skilled in the art will appreciate that an osmoprotectant confers resistance to water or salt stress when present in the cell in high amounts, and confers membrane stability in lower amounts. Osmoprotectants include sugars such as monosaccharides, disaccharides, oligosaccharides, polysaccharides, sugar alcohols, and sugar derivatives, as well as proline and glycine-betaine. A preferred embodiment of the invention is an osmoprotectant that is a sugar. Useful osmoprotectants include fructose, erythritol, sorbitol, dulcitol, glucoglycerol, sucrose, stachyose, raffinose, ononitol, mannitol, inositol, methyl-inositol, galactol, hepitol, ribitol, xylitol, arabitol, trehalose, and pinitol.

Genes which encode an enzyme that catalyzes the synthesis of an osmoprotectant include genes encoding mannitol dehydrogenase (Lee and Saier, J. Bacteriol., 153 (1982)) and trehalose-6-phosphate synthase (Kaasen et al., J. Bacteriol., 174, 889 (1992)). Through the subsequent action of native phosphatases in the cell or by the introduction and coexpression of a specific phosphatase into the nucleus, these introduced genes result in the accumulation of either mannitol or trehalose in the nucleus, respectively, both of which have been well documented as protective compounds able to mitigate the effects of stress. Mannitol accumulation in the nucleus of transgenic tobacco has been verified and preliminary results indicate that plants expressing high levels of this metabolite are able to tolerate an applied osmotic stress (Tarczynski et al., cited supra (1992), (1993)).

Also provided is an isolated transformed plant cell and an isolated transformed plant comprising said transformed cells, which cell and plant are substantially tolerant of or resistant to a reduction in water availability. The cells of the transformed monocot plant comprise a recombinant DNA sequence comprising a preselected DNA sequence encoding an enzyme which catalyzes the synthesis of an osmoprotectant. The preselected DNA sequence is present in the cells of the transformed plant and the enzyme encoded by the preselected DNA sequence is expressed in those cells to yield an amount of osmoprotectant effective to confer tolerance or resistance to those cells to a reduction in water availability that inhibits the growth of the corresponding untransformed plant cells. A preferred embodiment of the invention includes a transformed plant that has an

improved osmotic potential when the total water potential of the transformed plant approaches zero relative to the osmotic potential of a corresponding untransformed plant.

As used herein, a "preselected" DNA sequence is an exogenous or recombinant DNA sequence that encodes an enzyme which catalyzes the synthesis of an osmoprotectant, such as sugar. The enzyme preferably utilizes a substrate that is abundant in the plant cell. It is also preferred that the preselected DNA sequence encode an enzyme that is active without a co-factor, or with a readily available co-factor. For example, the mild gene of *E. Coli* encodes a mannitol-1-phosphate dehydrogenase (M1PD). The only co-factor necessary for the enzymatic activity of M1PD in plants is NADH and the substrate for M1PD in plants is fructose-6-phosphate. Both NADH and fructose-6-phosphate are plentiful in higher plant cells.

As used herein, "substantially increased" or "elevated" levels of an osmoprotectant in a transformed plant cell, plant tissue, plant part, or plant, are greater than the levels in an untransformed plant cell, plant part, plant tissue, or plant, i.e., one where the chloroplast genome has not been altered by the presence of a preselected DNA sequence. In the alternative, "substantially increased" or "elevated" levels of an osmoprotectant in a water-stressed transformed plant cell, plant tissue, plant part, or plant, are levels that are at least about 1.1 to 50 times, preferably at least about 2 to 30 times, and more preferably about 5-20 times, greater than the levels in a non-water-stressed transformed plant cell, plant tissue, plant part of plant.

As used herein, a plant cell, plant part, plant tissue or plant that is "substantially resistant or tolerant" to a reduction in water availability is a plant cell, plant part, plant tissue, or plant that grows under water-stress conditions, e.g., high salt, low temperatures, or decreased water availability, that normally inhibit the growth of the untransformed plant cell, plant tissue, plant part, or plant, as determined by methodologies known to the art. Methodologies to determine plant growth or response to stress include, but are not limited to, height measurements, weight measurements, leaf area, plant water relations, ability to flower, ability to generate progeny, and yield. For example, a stably transformed plant of the invention has a superior osmotic potential during a water deficit relative to the corresponding.

As used herein, an "exogenous" gene or "recombinant" DNA is a DNA sequence that has been isolated from a cell, purified, and amplified.

As used herein, the term "isolated" means either physically isolated from the cell or synthesized in vitro in the basis of the sequence of an isolated DNA segment.

As used herein, a "native" gene means a DNA sequence or segment that has not been manipulated in vitro, i.e., has not been isolated, purified, and amplified.

The invention also provides, preferably, a plastid vector that is capable of stably transforming and conferring drought resistance to tolerance to different plant species.

The invention provides a plastid vector comprising of a DNA construct. The DNA construct includes a 5' part of the plastid DNA sequence inclusive of a spacer sequence; a promoter that is operative in the plastid; heterologous DNA sequences comprising at least one gene of interest encoding a molecule; a gene that confers resistance to a selectable marker; a transcription termination region functional in the target plant cells; and a 3' part of the plastid DNA sequence inclusive of a spacer sequence. The molecule can be a peptide of interest. Preferably, the vector includes a ribosome binding site (rbs) and a 5' untranslated region (5'UTR). A promoter functional in green or non-green plastids is used in conjunction with the 5'UTR.

Further, the invention provides a heterologous DNA sequence, which codes for an osmoprotectant, such as the Yeast T6P synthase gene (TSP1 gene), the E. coli otsA gene. The invention also provides the psbA 3' region, which enhances the translation of foreign genes.

The invention provides a promoter is one that is operative in green and non-green plastids such as the 16SrRNA promoter, the psbA promoter, and the accD promoter.

The invention provides a gene that confers resistance, such as antibiotic resistance like the aadA gene or an antibiotic-free selectable marker such as BADH or the chlB gene, as a selectable marker.

All known methods of transformation can be used to introduce the vectors of this invention into target plant plastids including bombardment, PEG Treatment, Agrobacterium, microinjection, etc.

The invention provides transformed crops, like solanaceous plants that are either monocotyledonous or dicotyledonous. Preferably, the plants are those having economic value which are edible for mammals, including humans.

Any plant can be transformed to an osmprotectant-expressing plant in accordance of the inyention which can carry a helogerous DNA sequence which encodes a desired trait. The transformed osmoprotectant-expressing plant need not comprise such a trait other than the DNA sequence which encodes the osmoprotentant.

The invention provides plants that have been transformed via the chloroplast which

accumulate trehalose at an amount at least 17-fold higher than non-transformed plants which are drought resistant.

The invention provides plants that have been transformed via the chloroplast which has at least a seven-fold increase in TPS1 activity.

The invention provides plants that have been transformed via the chloroplast which, in the T_0 generation, display otherwise normal phenotype other than decreased growth and delayed flowing. The invention further provides that the T_1/T_2 generations of the transformed plants display no pleiotropic effects.

The invention provides the transformed chloroplasts of the target plants which contain high levels of trehalose.

The invention provides for chloroplast transformant seedlings which are drought resistant which are resistant to medium containing 3% to 6% PEG.

The invention provides a method to confer drought resistance to plants via chloroplast transformation with a universal chloroplast vector which contains a drought-resistant or osmoprotectant gene and the accumulation of high levels of trehalose in the chloroplast.

The invention provides a method to transform a target plant for expression of the TPS1 gene leading to accumulations of trehalose in the chloroplast of the plant cells and eliminating adverse pleiotropic effects.

The invention provides proof of integration of the heterologous DNA sequence into the chloroplast genome by PCR.

The invention provides an environmental friendly method of engineering drought resistance to plants through chloroplast transformation.

Yeast *trehalose phosphate synthase* (*TPS1*) gene was introduced into the tobacco chloroplast or nuclear genomes to study resultant phenotypes. PCR and Southern blots confirmed stable integration of *TPS1* into the chloroplast genomes of T_1, T_2 and T_3 transgenic plants. Northern blot analysis of transgenic plants showed that the chloroplast transformant expressed 16,966-fold more *TPS1* transcript than the best surviving nuclear transgenic plant. Although both the chloroplast and nuclear transgenic plants showed significant TPS1 enzyme activity, no significant trehalose accumulation was observed in T_0/T_1 nuclear transgenic plants whereas chloroplast transgenic plants showed 15-25 fold higher accumulation of trehalose than the best surviving nuclear transgenic plants. Nuclear transgenic plants (T_0) that showed significant amounts of trehalose accumulation showed

stunted phenotype, sterility and other pleiotropic effects whereas chloroplast transgenic plants (T_1, T_2, T_3) showed normal growth and no pleiotropic effects. Chloroplast transgenic plants also showed a high degree of drought tolerance as evidenced by growth in 6% polyethylene glycol whereas untransformed plants were bleached. After 7hr drying, chloroplast transgenic seedlings (T_1, T_3) successfully rehydrated while control plants died. There was no difference between control and transgenic plants in water loss during dehydration but dehydrated leaves from transgenic plants (not watered for 24 days) recovered upon rehydration while control leaves died. In order to prevent escape of drought tolerance trait to weeds and associated pleiotropic traits to related crops, it is desirable to genetically engineer crop plants for drought tolerance via the chloroplast genome instead of the nuclear genome.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. PCR analysis of control and chloroplast transformants. **A.** Map of pCt-TPS1, chloroplast transformation vector and primer landing sites. P denotes plus strand and M denotes minus strand. Please note that tRNA genes contain introns. **B.** 1% agarose gel containing PCR products using total plant DNA as template. M: 1 kb ladder; 1. N. *Nicotiana tabacum* Burley, untransformed control; Lanes 1, 3, 5: pCt basic vector transformants. 2, 4, 6: pCt-TPS1 transformants. **C.** Map of the nuclear expression vector pHGTPS1.

Figure 2. Southern blot analysis of control, T_1 and T_3 chloroplast transgenic plants. **A.** Site of integration of foreign genes into the chloroplast genome and expected fragment sizes in Southern blots. P1 is the 0.81kb BamH1-BglII fragment containing chloroplast DNA flanking sequences used for homologous recombination. P2 is the 1.5kb Xba1 Fragment containing the TPS1 coding sequence. **B.** Southern blot of DNA digested with BglII and hybridized with probes P1 or P2. Lanes: C, untransformed control; 1, T_1 generation chloroplast transformant; 2, T_3 generation chloroplast transformant.

Figure 3. Northern and western blot analyses of control, nuclear and chloroplast transgenic plants. **A, D** Western blots detected through chemiluminescence (100μg total protein per lane). **B, E** Northern blots detected using ^{32}P *TPS1* probe. **C, F** Ethidium bromide stained RNA gel before blotting (10μg total RNA loaded per lane). Panel **A, B, C**: T_0 nuclear and T_1 chloroplast transgenic plants. Lanes: 1. *N. t.* xanthi control; 2~5: T_0 nuclear transgenic plants. 2, X-113; 3. X-119; 4. X-121; 5. X-224; 6: *N.t.* Burley control; 7: chloroplast transgenic plant (T_1). Panel **D, E, F**: T_1 nuclear and T_2 chloroplast transgenic plants. Lanes: 1. *N. t* xanthi control; 2, 3: T_1 nuclear

transgenic plants 2, X-113; 3.X-119; 4: *N.t.* Burley control; 5: chloroplast transgenic plant (T_2).

Figure 4. Nuclear and chloroplast transgenic plants to illustrate pleiotropic effects. 1. *N. t* xanthi control; 2~5: T_0 nuclear transgenic plants 2, X-113; 3.X-121; 4. X-119; 5. X-224; 6, T_1 chloroplast transgenic plant; 7, N. t. Burley control.

Figure 5. Germination of T_1, T_2 and T_3 generation of chloroplast transformants and untransformed control on MS plate containing spectinomycin (500µg/ml).

Figure 6. Assay for drought tolerance on PEG. Four week old seedlings on MS medium containing 3% (**A, B**) or 6% (**C, D**) polyethylene glycol (MW 8,000). **A, C**: Control untransformed *N.t.* Burley. **B, D**: T_1 Chloroplast transgenic plants.

Figure 7. Dehydration/rehydration assay. Three week old seedlings from control and chloroplast transgenic lines germinated on agarose in the absence or presence of spectinomycin (500µg/ml) were air-dried at room temperature in 50% relative humidity. After 7 hrs drying, seedlings were rehydrated for 48 hrs by placing roots in MS medium. A, untransformed; B,C, T_1 and T_3 chloroplast transgenic lines.

Figure 8. Water loss assay. Detached leaves from mature plants at similar developmental stages were dried at room temperature in 25% relative humidity. Leaf weight during drying was recorded and shown as percentage of initial fresh weight.

Figure 9. Dehydration and rehydration of potted plants. Potted plants were not watered for 24 days and rehydrated for 24 hours. Arrows indicate fully dried leaves that either recovered or did not recover from dehydration. A, C: Control untransformed; B,D: chloroplast transgenic plants.

DETAILED DESCRIPTION OF THE INVENTION

This invention discloses a method of conferring drought tolerance to plants by transforming plants via the chloroplast with a vector that contains a DNA sequence encoding a gene of interest that protects against water stress. In the preferred embodiment of this invention, the vector used is the universal vector as described by Daniell in WO99/10513, which is incorporated herein by reference. Other vectors that are capable of chloroplast transformation such as pUC, pBR322, pBlueScript, pGem and others described in U.S. patent numbers 5,693,507 and 5,932,479 may be used. In the preferred embodiment of this invention, the osmoprotection is the yeast trehalose-6-phosphate synthase (TSP1). Other genes which are capable of conferring drought resistance or osmoprotection

may also be used.

Expression of yeast TPS1 in E. coli:

It is known that the yeast trehalose-6-phosphate synthase gene can be expressed in nuclear transgenic plants. Because chloroplasts are prokaryotic in nature, it is desirable to test expression levels of the eukaryotic yeast TPS1 gene in E coli. Because of the high similarity in the transcription and translation systems between E. coli and chloroplasts, expression vectors are. routinely tested in E. coli before proceeding with chloroplast transformation of higher plants. Therefore, the TPS1 gene from yeast was cloned into the E. coli expression vector pQE 30 (see Figure 1A for details of pQE-TPS1) and expressed in a suitable E. coli strain M15 (pREP4). SDS-PAGE as shown in Figure 1B shows the presence of TPS1 protein in crude cell extracts, even with Coomassie Blue stain (lane 1), indicating high levels of expression. Western blot analysis using TPS1 -antibody confirms the true identity of the expressed protein as shown in Figure 1B, lane 41. These results confirm that the codon preference of TPS1 is compatible for expression in a prokaryotic compartment. Hyper-expression also facilitated purification as shown in Figure 1, lanes 2.55 and preparation of polyclonal antibody for characterization of transgenic plants.

Chloroplast and nuclear expression vectors.

Having confirmed suitability for prokaryotic expression, the yeast TPS1 gene was inserted into the universal chloroplast expression vector pCt-TPS1 as shown in Figure 2B. This vector can be used to transform chloroplast genomes of several plant species because the flanking sequences are highly conserved among higher plants. This vector contains the 16SrRNA promoter (Prrn) driving the aadA (aminoglycoside 3"- adenylyl transferase) and TPS1 genes with the psbA 3' region (the terminator from a gene coding for photosystem II reaction center component) from the tobacco chloroplast genome. It is known that the 16SrRNA promoter is one of the strong chloroplast promoters and the psbA 3' region stabilized transcripts to avoid hyper-expression of TPS-1 and associated Pleiotropic effects. The yeast ribosme binding site (RBS) was used instead of the genome chloroplast RBS (GGAGG). This construct integrates both genes into the spacer region between the chloroplast transfer RNA genes coding for alanine and isoleucine within the inverted repeat (IR) region of the chloroplast genome by homologous recombination. For nuclear expression, the yeast TPS1 gene was inserted into the binary vector pHGTPS1 (Figure 2C), in which the TPS1 gene is driven by the CaMV 35S promoter and the hph gene is driven by the nopaline synthase promoter. The expression cassette is flanked by both the left and right T-DNA border sequences.

The binary vector pHGTPS1 was mobilized into the *Agrobacterium tumafaciens* strain LBA 4404 by electroporation. Transformed Agrobacterium strain was introduced into Nicotiana tabaccum var xanthi using the leaf disc transformation method. Ninety two independent TPS1 nuclear tranformants were obtained on hygromycin selection. Seventeen confirmed nuclear tranformants were analyzed by northern blots. Among tranformants showing various levels of transcripts, five tranformants with strong, moderate, weak, very weak and absence of transcripts were chosen for further characterization. For chloroplast transformation, green leaves of N. tabacum var. Burley were transformed with the chloroplast integration and expression vector by the biolistic process. Bombarded leaf segments were selected on spectinomycin/streptomycin selection medium. Integration of foreign gene into the chloroplast genome was determined by PCR screening of chloroplast tranformants, (Figure 2A). Primers were designed to eliminate mutants, nuclear integration and to determine whether the integration of foreign genes had occurred in the chloroplast genome at the directed site by homologous recombination. Primers 5P/5M land within the aadA gene and should generate a 0.4 kbp fragment if the aadA gene was present in transgenic plants and eliminates the possibility of mutation that could otherwise confer streptomycin/spectinomycin resistance. Figure 2A shows the presence of 0.4 kbp PCR product in plants transformed with the universal vector alone (pCt,) or the universal vector containing the TPS1 gene (pCt-TPS1), but not in control untransformed plants, confirming that these are transgenic plants and not mutants. The strategy to distinguish between nuclear and chloroplast transgenic plants was to land one primer (3P) on the native chloroplast genome adjacent to the point of integration and the second primer (3M) on the aadA gene. This primer set generated 1.6 kbp PCR product in chloroplast tranformants obtained with the universal vector (pCt) and the universal vector containing the TPS1 gene (pCt-TPS1). Because this product can not be obtained in nuclear transgenic plants, the possibility of nuclear integration can be eliminated. Another primer set was designed to test integration of the entire gene cassette. The presence of the expected size PCR products using 5P/5M confirms that the entire gene cassette has been integrated and that there has been no internal deletions or loop outs during integration via homologous recombination.

Determination of chloroplast integration, homoplasmy and copy number:

Since there are no significant differences in the level of foreign gene expression among different chloroplast transgenic lines, one line was chosen to generate subsequent generations ($T_1T_2T_3$). Southern blot analysis was performed using total DNA isolated from transgenic and wild

type tobacco leaves. Total DNA was digested with a suitable restriction enzyme. Presence of a BglII at the 3' end of the flanking 16S rRNA gene and the trnA intron allowed excision of predicted size fragments in the chloroplast tranformants and untransformed plants. To confirm foreign gene integration and homoplasmy, individual blots were probed with the chloroplast DNA flanking sequence (probe P1, Figure 2A). In the case of the *TPS1* integrated plastid tranformants (T_1T_2), the border sequence hybridized with 6.13 and 1.17 kbp fragments while it hybridized with a native 4.47 kbp fragment in the untransformed plants (Figure 2B). The copy number of the integrated *TPS1* gene was also determined by establishing homoplasmy in transgenic plants. Tobacco chloroplasts contain about 10,000 copies of chloroplast genomes per cell. If only a fraction of the genomes were transformed, the copy number should be less than 10,000. By confirming that the *TPS1* integrated genome is the only one present in transgenic plants, one could establish that the *TPS1* gene copy number could be as many as 10,000 per cell.

DNA gel blots were also probed with the *TPS1* gene coding sequence (probe P2) to confirm integration into the chloroplast genomes. In chloroplast transgenic plants (T_1T_3), the *TPS1* gene coding sequence hybridized with 6.13 and 1.17 kbp fragments which also hybridized with the border sequence in plastid transgenic lines (Figure 2B). This confirms that the tobacco tranformants indeed integrated the intact gene expression cassette into the chloroplast genome and that there has been no internal deletions or loop out during integration via homologous recombination.

Analysis of transcript level in nuclear and chloroplast tranformants:

For comparison of introduced gene expression between chloroplast and nuclear tranformants, northern blot analysis of transgenic tobacco at similar developmental stages was performed in T_1, T, and T_2 plants. As shown in Figure 3, quantification of transcription level showed that the chloroplast transformant (T2) expressed 16,960-fold (Figure 3E, lane 5) more TPS1 transcript than that of highly expressing nuclear (T_1) transformant (Figure 3E, lanes 2, 3). Similar results were obtained when T_1 chloroplast (Figure 3B, lane 7) and T_0 nuclear transgenic plants (Figure 38, lanes 2-5) were compared. This large difference in TPS1 expression between nuclear and chloroplast transgenic plants should be due to the presence of thousands of TPS1 gene copies in each cell of transgenic tobacco. Figure 3 (C, F) show ethidium bromide stained RNA gels before blotting; this confirms that equal amount of RNA (10 μg) was loaded in all lanes. It is remarkable that the 16SrRNA promoter is driving both genes very efficiently, eliminating the need for inserting additional promoters for the gene of interest.

Western blot analysis of nuclear and chloroplast tranformants:

Polyclonal antibodies raised against the TPS1 protein overexpressed and purified from E. coli (see experimental protocol) were used for immunoblotting (Figure 3A, D). A 60 kDa TPS1 polypeptide was detected in the T_0 nuclear (Figure 3A, lanes 2,3,5), T_1 nuclear (3D lanes 2,3) and T_1 plastid (Figure 3A, lane 7) and T_2 plastid (Figure 3D, lane .5) tranformants. However, no TPS1 was detected in the untransformed control (Figure 3A, lanes 1,6; 3D 1,4)) and transgenic plants which showed no TPS1 transcript (Figure 3A, lane 4). As anticipated, western blots showed only a five or ten fold increase in TPS1 protein in chloroplast over highly expressing nuclear transgenic plants. This is because of the fact that the chloroplast vector pCt-TPS1 was intentionally designed to lower translation by not inserting a chloroplast preferred ribosome binding site (GGAGG), so that transgenic plants are not killed by hyper-expression of TPS1. This level expression was adequate to compare trehalose accumulation in cytosolic and chloroplast compartments and observe resultant phenotypic / physiological changes. T_1 nuclear and T_2 chloroplast transgenic plants had higher levels of TPS1 protein; this may be due to homozygous *TPS1* alleles or homoplasmy.

Quantification of trehalose-6-phosphate and trehalose in tranformants:

Trehalose formation is a two step process, involving trehalose-6-phosphate synthase and trehalose 6-phosphate phosphatase. Trehalose-6-phosphate was not detected in all tested chloroplast and nuclear transformers even though the TPS2, trehalose-6-phosphate phosphatase that converts T6P to trehalose, was not introduced (Table 1). Conversion of T6P to trehalose should have been accomplished by endogenous tobacco trehalose phosphatase or by any non-specific endogenous phosphatase. Simultaneous expression of both enzymes in transgenic plants resulted only in marginal increase of trehalose accumulation in previous studies, confirming that it is adequate to express only TPS1. Leaf extracts from both nuclear and chloroplast transgenic plants catalyzed the synthesis of trehalose 6-phosphate from glucose-6-phosphate and UDP-glucose whereas untransformed tobacco had very low activity. T_0 Chloroplast and nuclear transgenic plants showed a 7-10 fold higher TPS1 activity than untransformed control plants. The amount of trehalose present in untransformed control plants and T_0 nuclear transgenic plants were similar whereas chloroplast transgenic plants accumulated a 17-25 fold mm trehalose than the best surviving nuclear transgenic plants (Table 1). T_1 nuclear transgenic plants accumulated less trehalose than control untransformed plants whereas T_1 chloroplast transgenic plants continued to accumulate high levels of trehalose (Table 1). Observation of comparable TPS1 activity in both nuclear and chloroplast transgenic plants but lack

of trehalose accumulation in nuclear transgenic planes indicates that trehalose may be degraded in the cytosol by trehalase but not in the chloroplast compartment. This is consistent with previous studies on inhibition of trehalase activity that resulted in trehalose accumulation in the cytosol.

Drought tolerance and pleiotropic effects:

Chloroplast and nuclear tranformants were examined for drought tolerance and pleiotropic effects. After six weeks of growth in vitro, rooted shoots were transferred to pots and grown in the greenhouse. TPS1 nuclear tranformants showed moderate to severe growth retardation, lancet-shaped leaves and infertility (Figure 4). The chloroplast tranformants (T_0) showed decreased growth rate and delayed flowering but all subsequent generations (T_1, T_2) showed similar growth rates and fertility as controls. The nuclear transgenic lines of stunted phenotype showed delayed flowering and produced fewer seeds compared to wild type or did not flower. This result is consistent with prior observations which demonstrated that E. coli otsA (TPS1) and S. cerevisiae TPS1 transgenic plants exhibited stunted plant growth and other pleiotropic effects. The nuclear transgenic line showing severe growth retardation did not flower. T_1 nuclear transgenic plants that survived showed no growth retardation and trehalose accumulation. Therefore, these plants could not be used for appropriate comparison with chloroplast transgenic plants. When the seeds of chloroplast transgenic plant (crossed between transgenic female and untransformed male) and wild type seeds were germinated on MS medium containing spectinomycin, all chloroplast transgenic progeny were spectinomycin resistant while all wild type seedlings were sensitive to spectinomycin (Figure 5).

Because TPS1 transgenic lines showed accumulation of trehalose, they were tested for drought tolerance. Seeds of chloroplast and nuclear transgenic plants were germinated on the MS medium containing polyethylene glycol. As shown in Figure 6, chloroplast transformant seedlings showed resistance to medium containing 3% and 6% PEG whereas control and nuclear transgenic seedlings exhibited severe dehydration, necrosis and severe growth retardation, ultimately resulting in death. Three-week-old seedlings were chosen to study drought tolerance by dehydration and subsequent rehydration. When seedlings were dried for 7 hours at room temperature in 50% relative humidity, they were all affected by dehydration. However, when dehydrated seedlings were rehydrated for 48 hours in MS medium, all chloroplast transgenic lines recovered while all control seedlings were bleached (Figure 7). Even the couple of control seedlings that partly survived (because of uneven drying of seedlings on filter papers) eventually died. These results suggest that the loss of water from TPS1 transgenic plants may not be

decreased but the ability to recover from drought was dramatically enhanced. This is consistent with existing understanding that trehalose functions by protecting biological membranes rather than regulating water potential (Iwahashi et al., 1995).

Mature leaves from fully-grown plants were tested for their ability to regulate water loss under drought conditions. When detached leaves were air dried, control and chloroplast transgenic plants lost water to the same extent (Figure 8). Control and chloroplast transgenic potted plants were not watered for 24 days. Again, both showed dehydration to the same extent (Figure 9A,B). However, upon rehydration, fully dehydrated leaves (indicated by arrows, Figure 9C,D) recovered in chloroplast transgenic plants but not in controls.

This invention is exemplified by the following non-limiting example:

EXAMPLE ONE

Plant, A. tumefaciens and E. coli culture: For transformation experiments, Nicotiana tabacum var. xanthi and Burley were grown in MS medium in the Magenta culture box (Sigma, USA). For drought tolerance assays of transgenic tobacco plants, the rooted young plants were transferred to pre-swollen Jiffy-7 peat pellets (Jiffy Products, Norway) inside the greenhouse. Plants used for enzyme assays were grown and kept in Magenta culture boxes. Seven or 8 leaf stage plants were used for enzyme assays. Two to three-week old young transgenic tobacco plants were used for stress analyses. (*Agrobacterium tumefaciens* strain LBA4404 was grown in the YEP medium at 29°C In a shaking incubator. Other E. coli strains were cultured and maintained as described in Sambrook et al.

Plasmid construction and antibody production: For hyper-expression of the TPS1 in E. Coli for antibody production, the yeast TPS1 gene was cloned into plasmid pQE30 (Qiagen) and subsequently transformed into E. coli strain M15 [pREP4]. The resulting E. coli transformant was grown at 37°C to an A_{600} of 0.5-0.8 and induced by 2mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1-5 hours. The induced cells were harvested and lysed by sonication. SDS-PAGE analysis showed the presence of TPS1 protein in crude cell extracts, even with Coomassie Blue stain, indicating high levels of expression. Western blot analysis using TPS1 antibody confirmed the true identity of the expressed protein (data not shown). The recombinant protein was purified using Ni^{2+} resin, using the procedures provided by the manufacturer. Affinity column purified recombinant protein was analyzed for purity by SDS-PAGE. Protein concentrations were determined using 'the Bio-Rad

(USA) protein assay kit with BSA as a standard. Polyclonal antibody was generated using the purified TPS1 protein by the Takara Shuzo Co. (Japan).

Vector construction for plant transformation: The yeast 1.537 kbp TPS1 gene was inserted into the Xbal site of pCt vector generating pCt-TPS1 (Figure 2B). For the nuclear transformation, the yeast TPSl gene was inserted into the pHGTPS1 vector in which the TPS1 gene is driven by the CaMV 35S promoter. The resulting vector confers hygromycin resistance because of the hygromycin phosphotransferase gene driven by the NOS promoter.

Chloroplast and nuclear transformation: For chloroplast transformation, particle bombardment was carried out using a helium driven particle gun, Biolistic PDH1000. Briefly, chloroplast vectors, pCt and pCt-TPS1 were delivered to tobacco leaves (Burley) using 0.6 μm gold microcarriers (Bio-Rad) at 1,100 psi with a target distance of 9 cm. For nuclear transformation, pHGTPS1 was mobilized into the *Acrobacterium tumefaciens* strain LBA4404 by electroporation using Gene Pulsar (Bio-Rad. USA). The resulting *Agrobacterium* strain was used in leaf disc transformation of wild type N. tabacum var. xanthi.

Chloroplast DNA isolation and PCR: Total DNA was extracted from leaves of wild type and transformed plants using CTAB extraction buffer described. PCR was carried out to confirm spectinomycin resistant chloroplast tranformants using Peltier Thermal Cycler PTC-200 (MJ Research, USA). Three primer sets, 2P(5'-GCGCCTGACCCTG AGATGTGGATCAT-3')-2M(5'-TGACTGCCCAACCTGAGAGCGGACA-3'), 3P(AAAACCCGTCCTCAGTTCGGATTGC)-3M(CCGCGTTGTTTCATCA AGCCTTACG) and -5P(CTGTAGAAGTCACCATTGTTGTGC), 5M(GTCCAAGAT AAGCCTGTCTAGCTTC) were used for the PCR. PCR reactions were carried out as described elsewhere (Daniell et al., 1998; Guda et al., 2000).

RNA isolation and Northern Slot analysis: Total RNA was extracted from transgenic tobacco plants using Tri Reagent (MRC, USA) following manufacturer's instruction. For northern blots, RNA samples (10 μg of total RNA per lane) were electrophoresed on a 1.5% agarose-MOPS gel containing formaldehyde. Uniform loading and integrity of RNAs were confirmed by examining the intensity of ethidium bromide bound ribosomal RNA bands under UV light. RNAs on the gel were transferred onto Hybond-N membrane (Amersham, USA). The membrane was hybridized to radiolabeled TPS1 probe and washed at 65°C in a solution of 0.2X SSC and 0. 1 % SDS for 20 min twice. The blot was exposed to an X-ray film at -70°C overnight. Transcripts were quantified using the BiolD++ program with Vilber Lourmat Image Analyzer (Bioprofil, France).

Western Blot analysis: Tobacco total protein extracts were prepared by modified methods described by Ausubel et al. The total extracts were fractionated on a 10% one-dimensional SDS-PAGE, transferred to Biotrace PDVF nitrocellulose membrane (Gelman Sciences, USA), and immunostained using Renaissance Western Blot Chemiluminescence Reagent (NEN Life Science Products, USA) according to manufacturer's instructions. Each lane was loaded with 100 μg of total protein. The primary antibody used was anti-TPS1 at a 5000-fold dilution. The secondary antibody was anti-rabbit IgG HRP conjugate at a 2000-fold dilution (Promega, USA).

Drought tolerance and biochemical characterization: For analyses of drought tolerance, 2-3 week old transgenic tobacco plants were used. Seeds of chloroplast and nuclear tranformants were germinated on MS plates containing 3% or 6% PEG (MW 8,000). TPS1 enzyme assay was performed spectrophometrically by the method described by Londesbrough and Vuorio. For quantitative determination of T6P and trehalose, carbohydrates were extracted from aerial parts of transgenic or wild type tobacco plants by treatment in 85% ethanol at 60°C for 1 hour. The amount of T6P and trehalose were measured by high-performance liquid chromatography (HPLC) on a Waters system equipped with a Waters High Performance Carbohydrate Column (4.6x250 mm) and a refractive index detector. The insoluble phase system was 75% acetanitrile-25% H_20 with a flow rate of 1.0 ml/min.

REFERENCES

Thevelein, J.M. & Hohmann, S. Trehalose synthase: guard to the gate of glycolysis in yeast? Trends in Bioscience. 20, 3-10 (1955).

Singer, M.A. & Lindquist, S. Thermotolerance in Saccharomyces cerevisiae: the Yin and Yang of trehalose. Trends in Biotech. 16, 460-468 (1998).

Elbein, A.D. The metabolism of a _-trehalose. Adv Carbohyd Chem Biochem. 30, 227-256 (1974).

Mackenzie, K.F., Singh, K.K. & Brwon, A.D. Water stress plating hypersensitivity of yeast: protective role of trehalose in Saccharomyces cerevisiae. J Gen Microbial. 134, 1661-1666 (1988).

DeVigilio, C., Hottinger, T., Dominguez, J., Boller, T. & Wiekman, A. The role of trehalose synthesis for the acquisition of thermotolerance in yeast 1. Genetic evidence that trehalose is a thermoprotectant. Eur J Biochem. 219, 179-186 (1994).

Sharma, S.C. A possible role of trehalose in osmotolerance & ethanol tolerance in Saccharomyces cerevisiae. Fems Microbiology Letters. 152, 11-15 (1997).

Crowe J.H., Hoekstra F.A. & Crowe L.M. Anhydrobiosys. Annu Rev Physiol. 54, 579-599 (1992).

Bianchi, G. Gamba, A. & Limiroli, R. The unusual sugar composition in leaves of the resurrection plant Myrothamnus flatbellifolia. Physiol Plantarum. 87, 223-226 (1993).

Drennan, P.M., Smith, M.T., Goldsworthy, D. & Van Staden, J. The occurrence of trehalose in the leaves of the desiccation-tolerant angiosperm Myrothamnus flabellifolius Welw. J. Plant Physiol. 142, 493-496 (1993).

Colaco, C. Sen. S. Thangavelu, M., Pinder, S. & Roser, B. Extraordinary stability of enzymes dried in trehalose: simplified molecular biology. Biol technology, 10, 1007-1011(1992).

Iwahashi, H., Obuchi, K., Fujii, S. & Komatsu, Y. The correlative evidence suggesting that trehalose stabilizes membrane-structure in the yeast Saccharomyces cerevisiae. Cell. Mol. Biol. 41, 763-769 (1995).

Holmstrom, K.O., Mantyla, M., Wekin, B., Mandal, A., Palva. E.T., Tunnela, O.E. & Londesborough, J. Drought tolerance in tobacco. Nature. 379, 683-684 (1996).

Goddijyn, O.J.M., Verwoerd, T.C., Voogd, E., Krutwagen, W.H.H., ce Graff, P.T.H.M., Poels, J., van Dun, K., Ponstein, A.S., Damm, B. & Pen, K. Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. Plant Physiol. 113, 181-190 (1997).

Romero, C., Belles, J.M., Vaya, J.L., Serrano, R. & Culianz-Macia, F.A. Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta. 201, 293-297 (1997).

Serrano, R., Culianz-Macia, F.A. & Moreno, V. Genetic engineering of salt & drought tolerance with yeast regulatory genes. Scientia Horticulture 78:261-269.

During K. Hippe S. Kreuzaler F, Schell J (1990) Synthesis and self-assembly of a functional monocional antibody in transgenic Nicotiana tabacum. Plant Molecular Biology. 15, 281-293 (1999).

Daniell, H. & Guda, C. Biopolymer production in microorganisms and plants. Chemistry and industry. 14, 555-560 (1997).

Nuccio, M.L., Rhodes, D., McNeil, S.D. & Hanson, A.D. Metabolic engineering of plants for osmotic stress resistance. Crrunt opinion in plant biology 2: 128-134 (1999).

Daniell, H. New tools for chloroplast genetic engineering. Nat. Biotechnol.. 17, 855-856. (1999).

Daniell, H. GM crops: Public perception and scientific solutions. Trends in Plant Science. 4, 467-469 (1999).

Daniell, H. Environmentally friendly approaches to genetic engineering. In vitro Cellular and Developmental Biology-Plant. 35, 361-368 (1999).

Daniell, H. Genetically modified food crops: current concerns and solutions for the next generation crops. Biotechnology and Genetic Engineering Reviews, 17, in press.

Hoyle, B. Canadian farmers seek compensation for genetic pollution. Nat. Biotechnol 17, 747-748 (1999).

Finnegan J. & McElroy, D. Transgene Inactivation: Plants fight back. Biotechnology. 12, 883-888 (1994).

Kota, M., Daniell, H., Varma, S., Garcynski, F., Gould. F. & Morar, W.J. Overexpression of Bacillius thuringiensis Cry2A protein in chloroplasts confers resistance to plants against susceptible and Bt resistant insects. Proc Natl Acad Sci USA. 96, 1840-1845 (1999).

Daniell, H., Data, R., Varma, S., Gray, S.&Lee, S.-B. Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nature Biotech. 16, 345-348. (1998).

Scott. S.E. & Wilkinson, M.J. Low probability of chloroplast movement from oilseed rape (Brassica napus) into wild Brassica napa. Nat. Biotechnol. 17: 390-392. (1999).

Brixey,. P. J. Guda, C. & Daniell, H. The chloroplast psbA promoter is more efficient in E.coli than the T7 promoter for hyper-expression of a foreign protein. Biotechnology letters. 19, 2395-399 (1997).

Guda, G., Lee, S-B., & Daniell, H. Stable expression of a biodegradable protein-based polymer in tobacco chloroplasts. Plant Cell Reports 18, (1999).

Daniell, H. Transformation and foreign gene expression in plants mediated by microprojectile bombardment. Meth. Mol. Biol. 62, 453-488 (1997).

Roser, B. & Colaco, C.A sweeter way to fresher food New Scientist. 138, 24-28 (1993).

Sambrook. J., Maniatis, T. & Fritsch, E.J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989).

Dellaporta, S.L., Wood, J. & Hicks, J.B. A plant DNA minipreparation: Version II. Plant Mol Biol Rep. 1, 19. (1983).

Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Sediman, J.G., Smith, J.A. & Struhl, K. Short protocols in molecular biology. Wiley and Sons, Inc. USA (1995).

Londesbrough, J. & Vuorio, O. Trehalose-6 phosphate synthase/phosphat-ase complex from baker's yeast: purification of a protelytically activated form. J. Gen. Microbiology. 137, 323-330 (1991).

Herbicide Resistance Crops, Agricultural, Environmental, Economic, Regulatory and Technical Aspects, Duke, S.O., edt., CRC Press, Inc. (1996).

Herbicide Resistance in Plants, Biology and Biochemistry, Powles, S.B., and Holtum, J.A.M., eds., CRC Press, Inc. (1994).

Peptides: Design, Synthesis, and Biological Activity, Basava, C. and Anantharamaiah, G.M., eds., Birkhauser Boston, 1994.

Protein Folding: Deciphering the Second Half of theGenetic Code, Gierasch, L.M., and King, J., eds., American Association For the Advancement of Science (1990).

What is claimed is:

1. An integration and expression plastid vector competent for stably transforming the plastid genome of which confer stress tolerance which comprises an expression cassette which comprises as operably joined components, a 5' part of the plastid DNA sequence inclusive of a spacer sequence, a promoter operative in said plastid, a selectable marker sequence, a DNA sequence encoding for an osmoprotectant, at least one restriction site for the insertion of a heterologous target DNA sequence, a transcription termination region functional in said plastid, and the 3' part of the plastid DNA sequence inclusive of a spacer sequence.

2. The vector of claim 1 further comprising a heterologous DNA sequence which codes for a molecule of interest that is inserted in one of the restriction sites.

3. The vector of claim 2 where the molecule of interest is a polypeptide.

4. A vector of claim 2 or 3, wherein said vector further comprises a ribosome binding site and a 5' untranslated region (5' UTR) to enhance expression.

5. A vector of claim 2, 3, or 4 wherein the osmoprotectant is selected from a group consisting of sugars, sugar alcohols, sugar derivates, and amino acids including proline and glycine-betaine.

6. A vector of claim 5 wherein the osmoprotectant is trehalose.

7. A vector of claim 5 wherein the trehalose is at least one of the complex TPS1, TPS2, TPS3 or TSL1.

8. The vector of claim 2, 3 or 4 wherein the osmoprotectant is selected from a group consisting of TSP1, *E. Coli* otsA, stachyose, and ononitol.

9. The vector of claim 5 wherein the osmoprotectant is a sugar.

10. The vector of claim 9, wherein the sugar is a monosacharide including but not limited to fructose.

11. The vector of claim 9, wherein the sugar is a disaccharide including but not limited to sucrose.

12. The vector of claim 9, wherein the sugar is a trisaccharide including but not limited to raffinose.

13. The vector of claim 9 wherein the sugar is dulcitol.

14. The vector of claim 5 wherein the osmoprotectant is a sugar alcohol.

15. The vector of claim 14 wherein the sugar alcohol is a polyhyric alcohol.

16. The vector of claim 15 wherein the polyhyric alcohol is a trihydric alcohol including but not limited to glucoglycerol.

17. The vector of claim 15 wherein the polyhyric alcohol is a tetrahydric alcohol including but not limited to erythritol.

18. The vector of claim 15 wherein the polyhyric alcohol is a hexahydric alcohol including but not limited to mannitol or sorbitol.

19 A vector of claim 2, 3 or 4 wherein at least one DNA encodes a component of trehalose synthase that is under the control of a promoter to produce a transgenic plant.

20. The vector of claim 19 wherein the promoter is constitutive.

21. The vector of claim 19 wherein the promoter is tissue specific, light-induced, or stress-induced.

22. A stably transformed plant which has been transformed by the vector of any one of claims 2-21, wherein the transformed plant is more tolerant of stresses selected from a group consisting of water-deprivation, freezing, salt, heat and cold than is the untransformed plant.

23. The plant of claim 22 wherein the plant does not include target DNA.

24. A stably transformed plant of claim 22, or the progeny thereof including seeds, wherein said plant display no negative pleiotropic effects.

25. A transgenic plant of any one of claims 22-25, wherein the plant is a transgenic plant which is morphologically indistinguishable from an untransformed plant.

26. A transgenic plant of any one of claims 22-25, wherein the plant is a solanaceous plant edible for a mammal.

27. A transgenic plant of any one of claims 22-25, wherein the plant is a crop plant edible for a mammal.

28. A transgenic plant of either claim 26 or 27, wherein the mammal is a human.

29. A transgenic plant of any one of claims 22-25, wherein the plant is a monocotyledonous plant selected from the group of rice, wheat, grass, rye, barley, oat, or maize.

30. A transgenic plant of any one of claims 22-25, wherein the plant is a dicotyledonous plant selected from the group of soybean, peanut, grape, sweet potato, pea, canola, tobacco, tomato or cotton.

31. A transgenic plant of any one of claims 22-25, wherein the plant is tobacco, tomato, potato, rice, brassica, cotton, maize or soybean.

32. A method of conferring drought resistance to plants, said method comprising introducing into the plastid of plant species that are susceptible to water stress, an expression cassette which comprises as operably joined components, a 5' part of the plastid DNA sequence inclusive of a spacer sequence, a promoter operative in said plastid, a DNA sequence encoding a gene which confers osmoprotection, a heterologous DNA sequence encoding a molecule of interest, a selectable marker sequence, a transcription termination region functional in said plastid, and a 3' part of the plastid DNA sequence inclusive of a spacer sequence.

33. The method of claim 32, wherein said method further comprises culturing said plant in a plant growth medium containing an effective amount of polyethylene glycol (PEG) for selection, and selecting transformed plant cells capable of growth in said plant growth medium.

34. The method of claim 33, wherein said method further comprises regenerating the selected transformed plant cells into stable transgenic plants.

35. A method of increasing trehalose accumulation in plant cells thereby conferring osmotic stress resistance to said plant cells, where said method comprises introducing to the plastid of plant species that are susceptible to osmotic stress an expression cassette which comprises as operably joined components, a 5' part of the plastid DNA sequence inclusive of a spacer sequence, a promoter operative in said plastid, a DNA sequence encoding the Yeast T6P synthase (TSP) gene which confers drought resistance, a heterologous DNA sequence encoding a molecule of interest, a selectable marker sequence, a transcription termination region functional in said plastid, and a 3' part of the plastid DNA sequence inclusive of a spacer sequence.

36. The method of claim 35, wherein said method further comprises culturing said plant in a plant growth medium containing an effective amount of polyethylene glycol (PEG) for selection, and selecting transformed plant cells capable of growth in said plant growth medium.

37. The method of claim 36, wherein said method further comprises regenerating the selected transformed plant cells into stable transgenic plants.

38. The vector of any one of claims 1-21, wherein said plastid is a chloroplast.

39. The vector of claim 38, wherein the vector is a universal chloroplast vector.

40. The methods of any one of claims 32-37, wherein the plastid is a chloroplast.

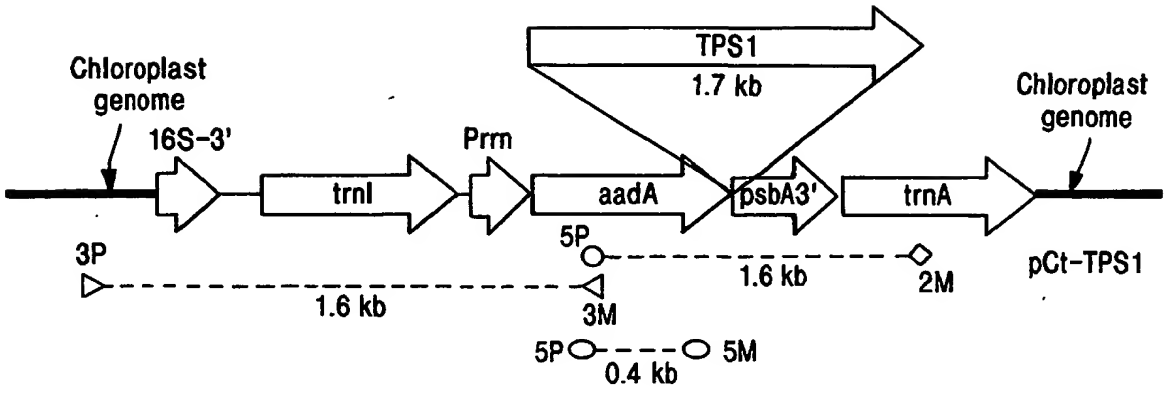


FIG. 1A

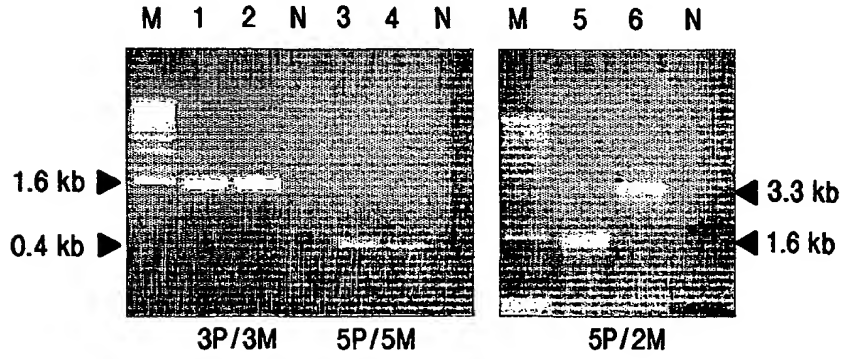


FIG. 1B

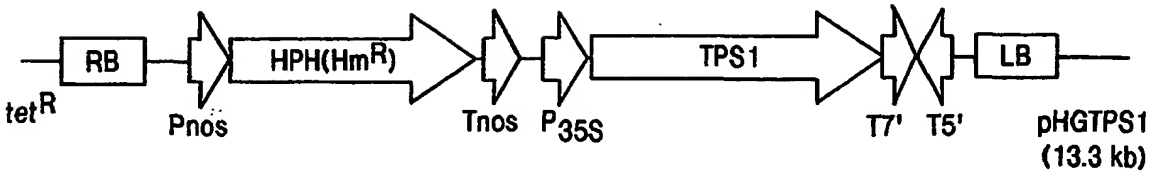


FIG. 1C

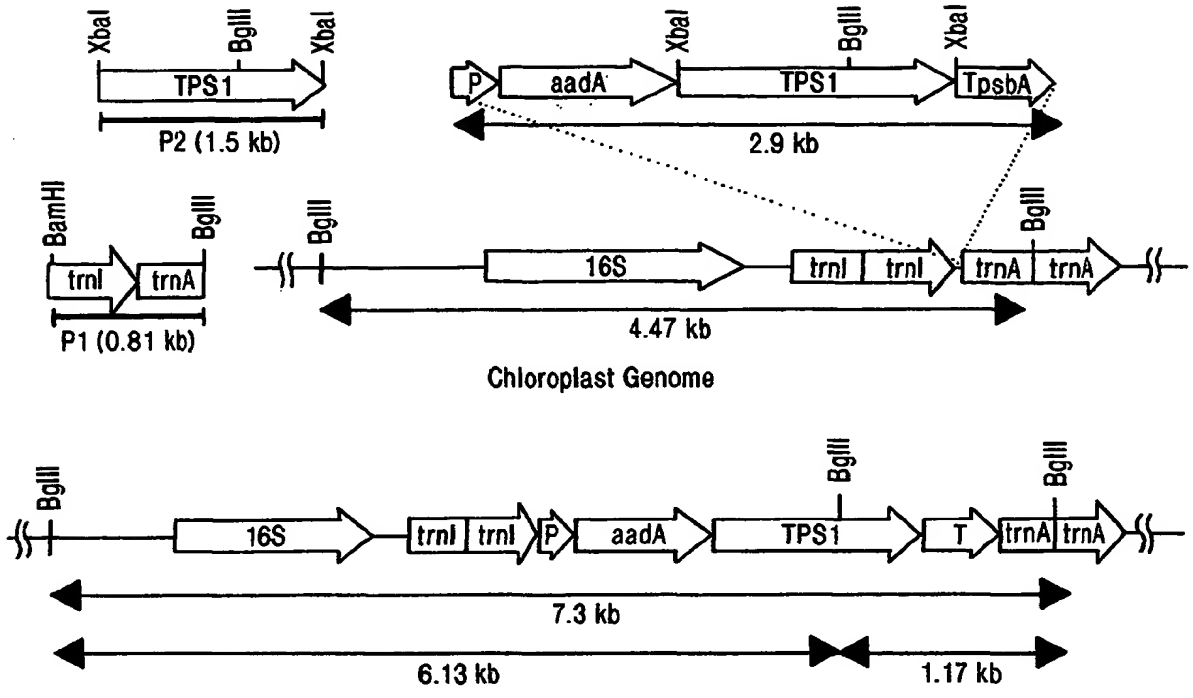

XbaI
BglII
XbaI
TPS1
P2 (1.5 kb)
P
aadA
XbaI
BglII
TPS1
XbaI
TpsbA
2.9 kb
BamHI
BglII
trnI
trnA
P1 (0.81 kb)
BglII
16S
trnI
trnI
BglII
trnA
trnA
4.47 kb
Chloroplast Genome
BglII
16S
trnI
trnI
P
aadA
BglII
TPS1
T
BglII
trnA
trnA
7.3 kb
6.13 kb
1.17 kb
Integration into Chloroplast genome

FIG. 2A

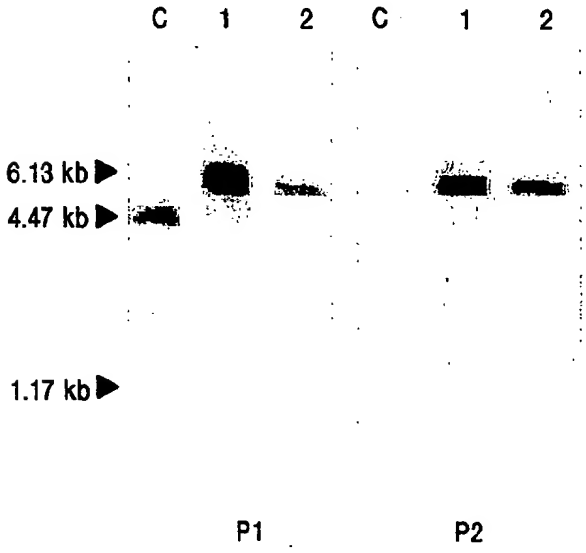

C
1
2
C
1
2
6.13 kb
4.47 kb
1.17 kb
P1
P2

FIG. 2B

FIG. 3A

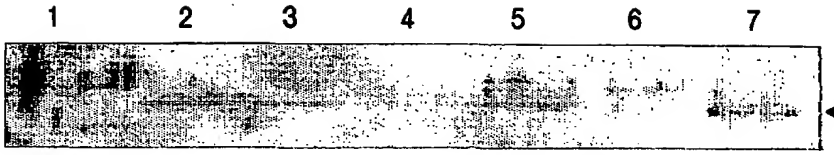


FIG. 3B

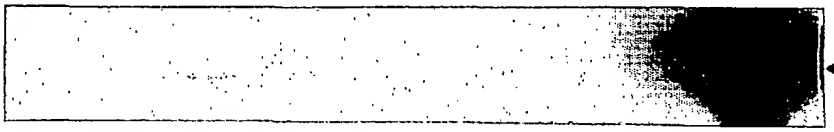


FIG. 3C

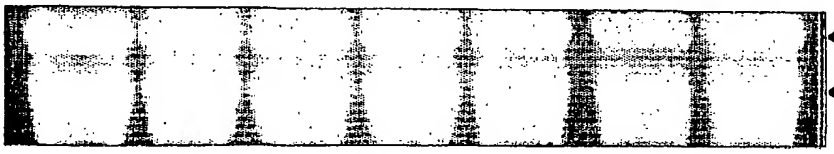


FIG. 3D

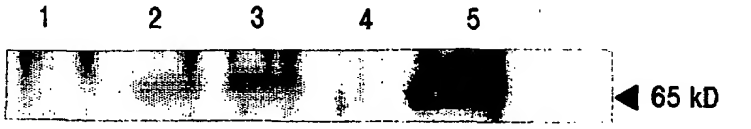


FIG. 3E

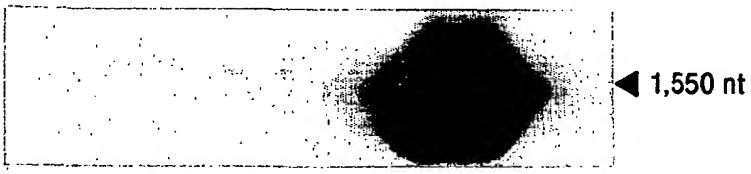
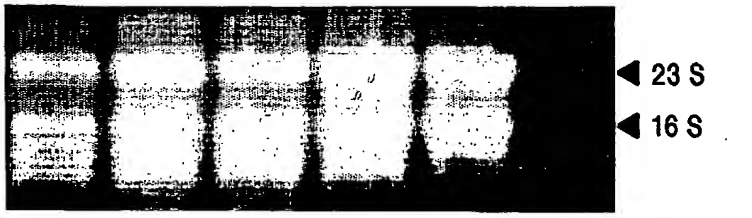


FIG. 3F



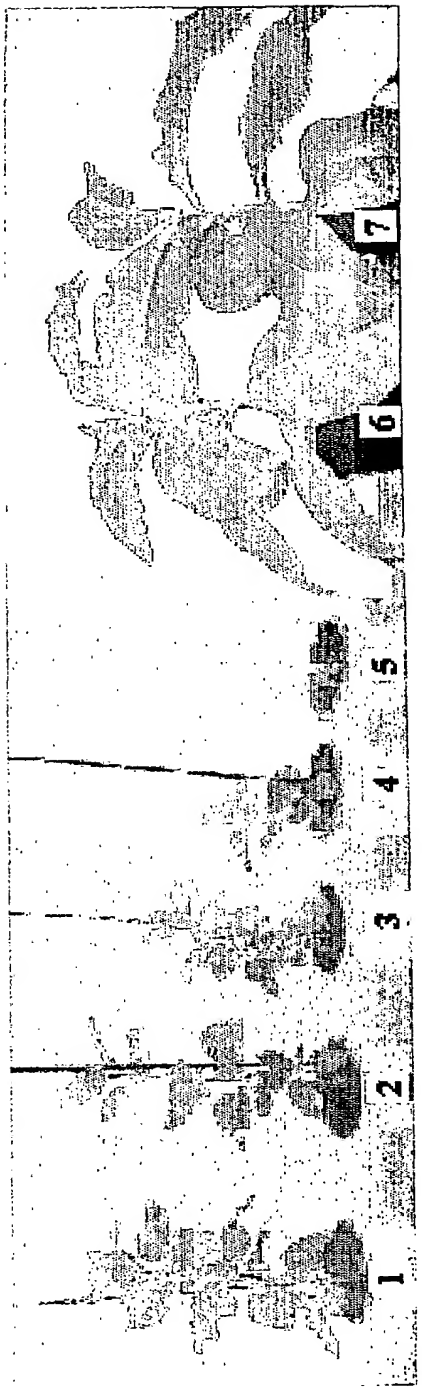


FIG. 4

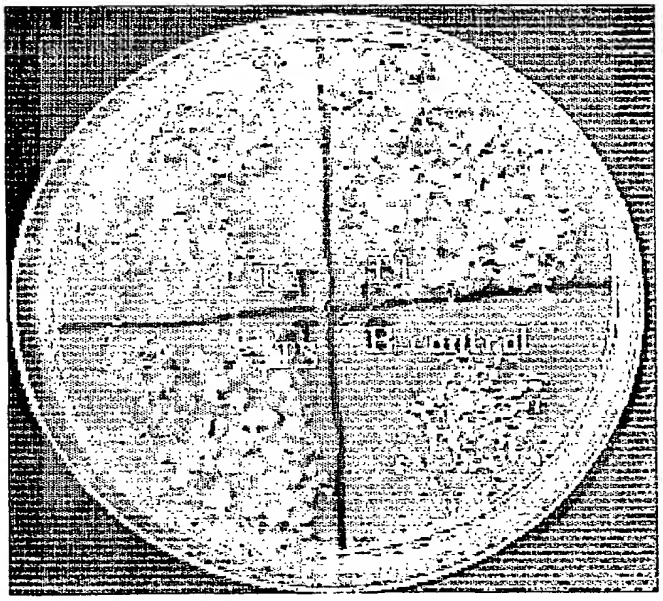


FIG. 5

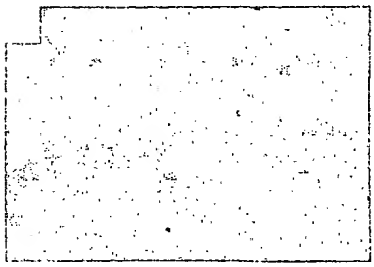


FIG. 6A

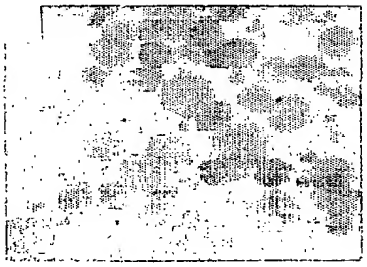


FIG. 6B

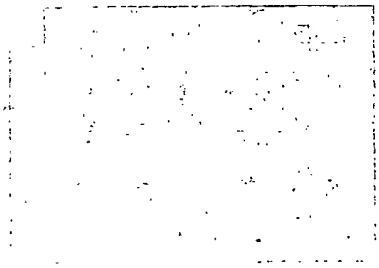


FIG. 6C

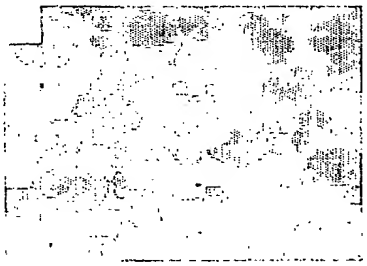


FIG. 6D

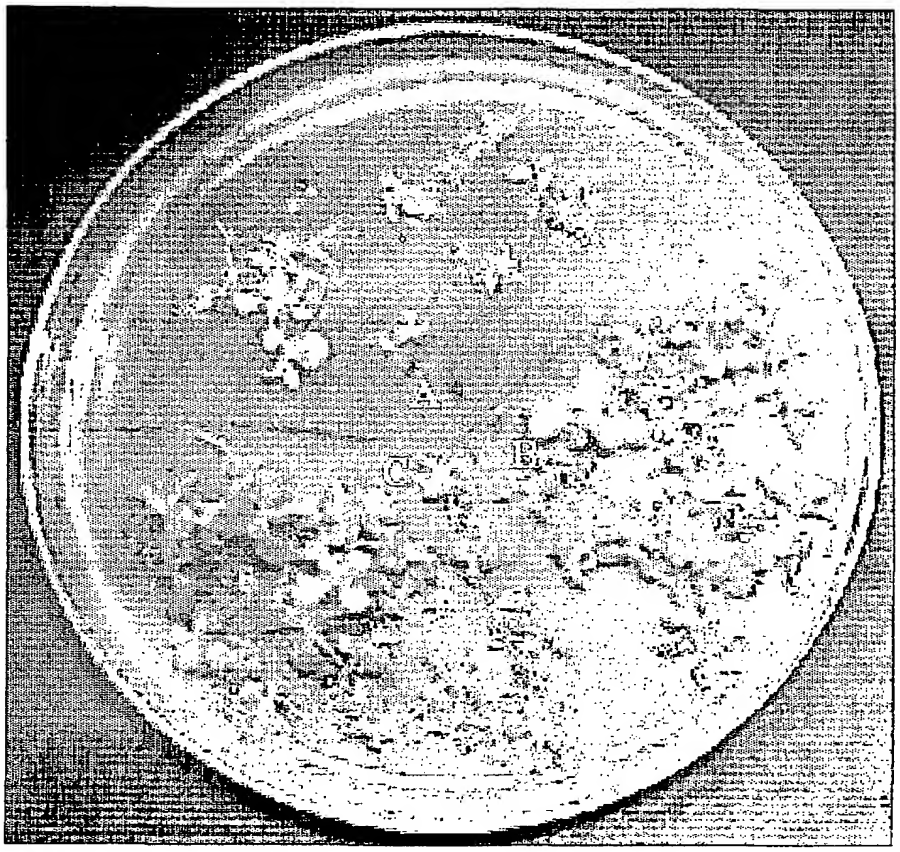


FIG. 7

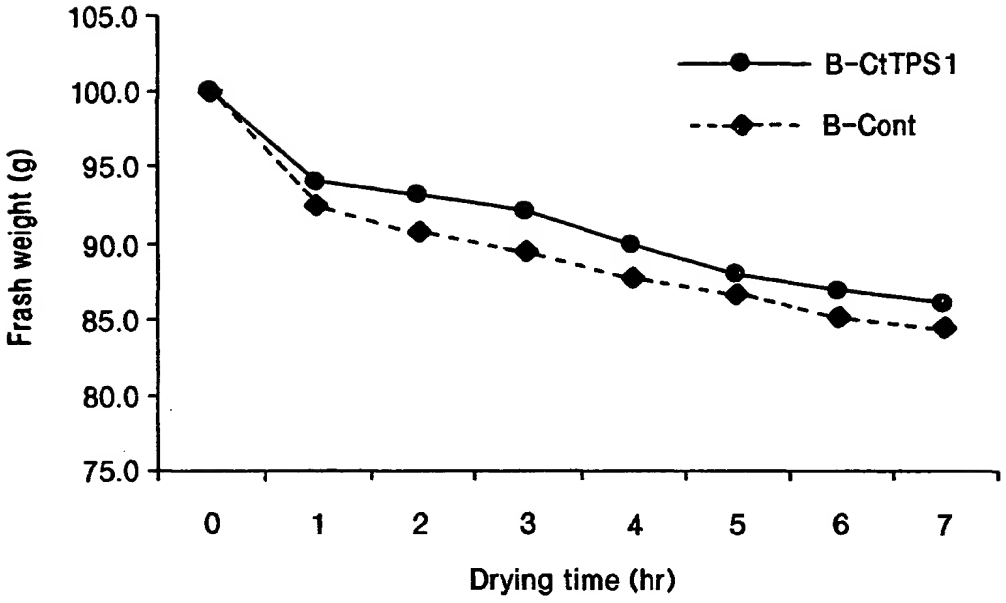

105.0
100.0
95.0
90.0
85.0
80.0
75.0
Frash weight (g)
0
1
2
3
4
5
6
7
Drying time (hr)
B-CtTPS1
B-Cont

FIG. 8

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06271

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 5/10, 15/82, 5/04; A01H 4/00

US CL :800/278, 284, 288, 289; 435/320.1, 468, 410, 419, 430, 431

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/278, 284, 288, 289; 435/320.1, 468, 410, 419, 430, 431

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 99/46370 (NOVARTIS AG) 16 September, 1999, pages 60-72, page 32, lines 21-23.	1-4, 32-34, 40 35-37
Y	US 5,693,507 A (DANIELL et al) 02 December 1997, col. 13, lines 14-40.	1-4, 32-37, 40
Y	US 5,792,921 A (LONDESBOROUGH et al) 11 August, 1998, col. 47, line 55, to col. 52, line 22.	1-4, 32-27, 40
Y	US 5,563,324 A (TARCZYNSKI et al) 08 October 1996, col. 9, lines 7-22, col. 10, line 49, to col. 11, line 3, col. 15, line 39, to col. 16, line 20, claims 1-13.	1-4, 32-34, 40

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 APRIL 2001	Date of mailing of the international search report 30 APR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE R. KUBELIK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06271

C.(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,780,709 A (ADAMS et al) 14 July 1998, col. 36, line 64 to col. 37, line 4; col. 51, lines 4-28; col. 52, line 51 to col. 56, line 20; claims 1-24.	1-4, 32-34, 40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06271

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-31 and 38-39
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment f additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06271

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

AGRICOLA, BIOSIS, CAPLUS, USPAT, EPO, JPO, DERWENT
Search terms: Trehalose, (plastid or chloroplast), (((water or drought or stress) and (resistan? or toleran?)) or osmotoleran? or osmoresistan?)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: GERARD J. WEISER
1600 MARKET STREET, SUITE 3600
PHILADELPHIA, PA 19103-7286

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing *(day/month/year)* 30 APR 2001
Applicant's or agent's file reference 1466-PCT-00	**FOR FURTHER ACTION** **See paragraphs 1 and 4 below**
International application No. PCT/US01/06271	International filing date *(day/month/year)* 28 FEBRUARY 2001
Applicant AUBURN UNIVERSITY	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE R. KUBELIK Telephone No. (703) 308-0196

Form PCT/ISA/220 (July 1998)*

(See notes on accompanying sheet)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 1466-PCT-00	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US01/06271	International filing date *(day/month/year)* 28 FEBRUARY 2001	(Earliest) Priority Date *(day/month/year)* 29 FEBRUARY 2000
Applicant AUBURN UNIVERSITY		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
   [ ] contained in the international application in written form.
   [ ] filed together with the international application in computer readable form.
   [ ] furnished subsequently to this Authority in written form.
   [ ] furnished subsequently to this Authority in computer readable form.
   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
2. [X] **Certain claims were found unsearchable** (See Box I).
3. [ ] **Unity of invention is lacking** (See Box II).
4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established by this Authority to read as follows:
5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the **drawings** to be published with the abstract is Figure No. _____
   [ ] as suggested by the applicant.
   [ ] because the applicant failed to suggest a figure.
   [ ] because this figure better characterizes the invention.
   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06271

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-31 and 38-39
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06271

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 5/10, 15/82, 5/04; A01H 4/00
US CL :800/278, 284, 288, 289; 435/320.1, 468, 410, 419, 430, 431

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/278, 284, 288, 289; 435/320.1, 468, 410, 419, 430, 431

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 99/46370 (NOVARTIS AG) 16 September, 1999, pages 60-72, page 32, lines 21-23.	1-4, 32-34, 40 35-37
Y	US 5,693,507 A (DANIELL et al) 02 December 1997, col. 13, lines 14-40.	1-4, 32-37, 40
Y	US 5,792,921 A (LONDESBOROUGH et al) 11 August, 1998, col. 47, line 55, to col. 52, line 22.	1-4, 32-27, 40
Y	US 5,563,324 A (TARCZYNSKI et al) 08 October 1996, col. 9, lines 7-22, col. 10, line 49, to col. 11, line 3, col. 15, line 39, to col. 16, line 20, claims 1-13.	1-4, 32-34, 40

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 APRIL 2001	Date of mailing of the international search report 30 APR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE R. KUBELIK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06271

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,780,709 A (ADAMS et al) 14 July 1998, col. 36, line 64 to col. 37, line 4; col. 51, lines 4-28; col. 52, line 51 to col. 56, line 20; claims 1-24.	1-4, 32-34, 40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06271

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

AGRICOLA, BIOSIS, CAPLUS, USPAT, EPO, JPO, DERWENT
Search terms: Trehalose, (plastid or chloroplast), (((water or drought or stress) and (resistan? or toleran?)) or osmotoleran? or osmoresistan?)

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under Article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

The statement should be brief, it should not exceed 500 words if in English or if translated into English.

It should not be confounded with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It should not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

In what language ?

The amendments must be made in the language in which the international application is published. The letter and any statement accompanying the amendments must be in the same language as the international application if that language is English or French; otherwise, it must be in English or French, at the choice of the applicant.

Consequence if a demand for international preliminary examination has already been filed ?

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase ?

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

HOME COPY

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No. PCT/US 01/06271

International Filing Date 28 FEB 2001 (28.02.01)

Name of Receiving Office and "PCT International Application" PCT INTERNATIONAL APPLICATION RO/US

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 1466-PCT-00

Box No. I TITLE OF INVENTION

Genetic Engineering of Drought Tolerance Via a Plastid Genome

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Auburn Uniiversity
309 Samford Hall,
Auburn University, AL 36849, US

☐ This person is also inventor.

Telephone No. 334-844-4977

Facsimile No. 334-844-5963

Teleprinter No.

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

University of Central Florida
4000 Central Florida Blvd.,
Orlando, FL 32816, US

This person is:

☒ applicant only

☐ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☒ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

WEISER, GERARD J.
1600 Market Street, Suite 3600,
Philadelphia, PA 19103-7286, US

Telephone No. 215-751-2427

Facsimile No. 215-568-6946

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Sheet No. 2

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

DANIELL, Henry
1255 Marina Point, #315
Casselberry, FL 32707, US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☒ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

DELETED BY RO/US

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

LEE, Seung-Bum
12722 Research Parkway
Orlando, FL 32826, US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☒ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: KR

State *(that is, country)* of residence: [KR] US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

RO/US

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

BYUN, Myung Ok
12722 Research Parkway
Orlando, FL 32826, US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☒ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: KR

State *(that is, country)* of residence: [KR] US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked):*

Regional Patent

☒ **AP ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ **EA Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian

☒ **EP European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ **OA OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line):*

☒	AE	United Arab Emirates	☒	LC	Saint Lucia
☒	AG	Antigua and Barbuda	☒	LK	Sri Lanka
☒	AL	Albania	☒	LR	Liberia
☒	AM	Armenia	☒	LS	Lesotho
☒	AT	Austria	☒	LT	Lithuania
☒	AU	Australia	☒	LU	Luxembourg
☒	AZ	Azerbaijan	☒	LV	Latvia
☒	BA	Bosnia and Herzegovina	☒	MA	Morocco
☒	BB	Barbados	☒	MD	Republic of Moldova
☒	BG	Bulgaria	☒	MG	Madagascar
☒	BR	Brazil	☒	MK	The former Yugoslav Republic of Macedonia
☒	BY	Belarus	☒	MN	Mongolia
☒	BZ	Belize	☒	MW	Malawi
☒	CA	Canada	☒	MX	Mexico
☒	CH and LI	Switzerland and Liechtenstein	☒	MZ	Mozambique
☒	CN	China	☒	NO	Norway
☒	CR	Costa Rica	☒	NZ	New Zealand
☒	CU	Cuba	☒	PL	Poland
☒	CZ	Czech Republic	☒	PT	Portugal
☒	DE	Germany	☒	RO	Romania
☒	DK	Denmark	☒	RU	Russian Federation
☒	DM	Dominica	☒	SD	Sudan
☒	DZ	Algeria	☒	SE	Sweden
☒	EE	Estonia	☒	SG	Singapore
☒	ES	Spain	☒	SI	Slovenia
☒	FI	Finland	☒	SK	Slovakia
☒	GB	United Kingdom	☒	SL	Sierra Leone
☒	GD	Grenada	☒	TJ	Tajikistan
☒	GE	Georgia	☒	TM	Turkmenistan
☒	GH	Ghana	☒	TR	Turkey
☒	GM	Gambia	☒	TT	Trinidad and Tobago
☒	HR	Croatia	☒	TZ	United Republic of Tanzania
☒	HU	Hungary	☒	UA	Ukraine
☒	ID	Indonesia	☒	UG	Uganda
☒	IL	Israel	☒	US	United States of America
☒	IN	India	☒	UZ	Uzbekistan
☒	IS	Iceland	☒	VN	Viet Nam
☒	JP	Japan	☒	YU	Yugoslavia
☒	KE	Kenya	☒	ZA	South Africa
☒	KG	Kyrgyzstan	☒	ZW	Zimbabwe
☒	KP	Democratic People's Republic of Korea			
☒	KR	Republic of Korea			
☒	KZ	Kazakhstan			

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

☒ CO Columbia

RO/US

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet need not be included in the request.*

*1. If, in any of the Boxes, **the space is insufficient** to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America**: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication **"patent of addition,"** or **"certificate of addition,"** or if, in Box No. V, the name of the United States of America is accompanied by an indication **"continuation"** or **"continuation-in-part"**: in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI) , the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed**: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, **the earlier application is an ARIPO application**: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudical disclosures or exceptions to lack of novelty" and furnish that statement below.*

Continuation of Box IV:

T. Daniel Christenbury	Reg. No. 31,750	Guy T. Donatiello	Reg. No. 33,167
Paul A. Taufer	Reg. No. 35,703	James A. Drobile	Reg. No. 19,690
Robert A. McKinley	Reg. No. 43,793	Austin R. Miller	Reg. No. 16,602
Sharon Fenick	Reg. No. 45,269	Stewart M. Wiener	Reg. No. 46,20
Joan T. Kluger	Reg. No. 38,940	Michael A. Patane	Reg. No. 42,982
Felicity Rowe	Reg. No. 47,042	Sharon Fenick	Reg. No. 45,269
Stephenie Yeung	Reg. No. P48,052		

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 29/02/2000 29 FEB 00	60/185,658	US		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): (1)

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA/US

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST: LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	:	5
description (excluding sequence listing part)	:	21
claims	:	3
abstract	:	1
drawings	:	9
sequence listing part of description	:	
Total number of sheets	:	39

This international application is **accompanied** by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☒ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: POSTCARD

Figure of the drawings which should accompany the abstract: 4A

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Date: 2/28/2001

Gerard J. Weiser

GERARD J. WEISER (28.02.01)

For receiving Office use only

1. Date of actual receipt of the purported international application: JC09 Rec'd PCT/PTO 28 FEB 2001
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA/US
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International application No. PCT/US 01/06271

Date stamp of the receiving Office 28 FEB 2001 (28.02.01)

Applicant's or agent's file reference: **1466-PCT-00**

Applicant: **Genetic Engineering of Drought Tolerance Via a Plastid Genome**

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE	240.00 T	240
2. SEARCH FEE	700.00 S	700

International search to be carried out by _____

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 39 sheets.

first 30 sheets	b1	382
9 remaining sheets x $9.00 additional amount =	81.00 b2	81
Add amounts entered at b1 and b2 and enter total at B	81.00 B	463

Designation Fees

The international application contains 87 designations.

6 number of designation fees payable *(maximum 6)* x 82.00 amount of designation fee =	492.00 D	492
Add amounts entered at B and D and enter total at I	573.00 I	955

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the

4. FEE FOR PRIORITY DOCUMENT *(if applicable)*	15.00 P	15
5. TOTAL FEES PAYABLE Add amounts entered at T, S, I and P, and enter total in the TOTAL box	1,528.00 TOTAL	1910

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☐ authorization to charge deposit account (see below)	☐ bank draft	☐ coupons
☒ cheque	☐ cash	☐ other *(specify)*:
☐ postal money order	☐ revenue stamps	

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ US

☐ is hereby authorized to charge the total fees indicated above to my deposit account.

☒ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

Deposit Account No.	Date *(day/month/year)*	Signature
13-3405	28/2/2001	Leland J. Weiss

PCT

GENERAL POWER OF ATTORNEY

(for several international applications filed under the Patent Cooperation Treaty)

(PCT Rule 90.5)

The undersigned person(s):
(Family name followed by given name: for a full legal entity, full official designation. The address must include postal code and name of country.)

Auburn University, 309 Samford Hall, Auburn University, AL 36849, US

hereby appoint(s) the following person as: ☒ agent ☐ common representative

Name and address
(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country.)

WEISER, Gerard J.	Reg. No. 19,783	Christenbury, T. Daniel	Reg. No. 31,750
Miller, Austin R.	Reg. No. 18,602	Donatiello, Guy T.	Reg. No. 33,187
Drobile, James A.	Reg. No. 16,890	McKinley, Robert A.	Reg. No. 43,793.
Miller, Austin R.	Reg. No. 16,602	Fenick, Sharon	Reg. No. 45,269
Kluger, Joan T.	Reg. No. 38,940	Patane, Michael A.	Reg. No. 42,982
Wiener, Steward M.	Reg. No. 46,201	Rowe, Felicity	Reg. No. 47,042
Yeung, Stephanie	Reg. No. P48,052		

to represent the undersigned before

☒ all the competent International Authorities

☐ the International Searching Authority only

☐ the International Preliminary Examining Authority only

in connection with any and all international applications filed by the undersigned with the following Office

US as receiving Office

and to make or receive payments on behalf of the undersigned.

Signature(s) *(where there are several persons, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading this power):*

By: C Moriarty

Printed Name: C. Michael Moriarty

Title: Associate Provost and Vice President for Research

Date: 2/26/01

PCT

GENERAL POWER OF ATTORNEY

(for several international applications filed under the Patent Cooperation Treaty)

(PCT Rule 90.5)

The undersigned person(s):
(Family name followed by given name; for a full legal entity, full official designation. The address must include postal code and name of country.)

University of Central Florida, 4000 Central Florida Boulevard, Orlando, Florida 32816, US

hereby appoint(s) the following person as: ☒ agent ☐ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

WEISER, Gerard J.	Reg . No. 19,763	Christenbury, T. Daniel	Reg. No. 31,750
Miller, Austin R.	Reg. No. 16,602	Donaliello, Guy T.	Reg. No. 33,167
Drobile, James A.	Reg. No. 19,690	McKinley, Robert A.	Reg. No. 43,793.
Miller, Austin R.	Reg. No. 16,602	Fenick, Sharon	Reg. No. 45,269
Kluger, Joan T.	Reg. No. 38,940	Patane, Michael A.	Reg. No. 42,982
Wiener, Stewart M.	Reg. No. 46,201	Rowe, Felicity	Reg. No. 47,042
Yeung, Stephenie	Reg. No. P48,052		

to represent the undersigned before

☒ all the competent International Authorities

☐ the International Searching Authority only

☐ the International Preliminary Examining Authority only

in connection with any and all international applications filed by the undersigned with the following Office

US as receiving Office

and to make or receive payments on behalf of the undersigned.

Signature(s) *(where there are several persons, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading this power):*

By: _____

Printed Name: Thomas O'Neil

Title: Director of Sponsored Research

Date: 2/26/01

Form PCT/Model of general power of attorney (for several international applications) (July 1992) LegalStar 1997, Form PCTM2

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
1466-PCT-00		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US01/06271	28 FEBRUARY 2001	29 FEBRUARY 2000
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant AUBURN UNIVERSITY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   [X] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [X] Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [ ] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21 SEPTEMBER 2001	07 MAY 2002
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ANNE R. KUBELIK
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US01/06271

I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed

☒ the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the claims:
pages (See Attached) , as originally filed
pages _____ , as amended (together with any statement) under Article 19
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the drawings:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the sequence listing part of the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/~~fig~~ NONE

5. ☒ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 5-31 and 38-39

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 5-31 and 38-39 are so unclear that no meaningful opinion could be formed *(specify)*.

because they are multiple dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 5-31 and 38-39 .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US01/06271

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	33-37	YES
	Claims	1-4, 32 and 40	NO
Inventive Step (IS)	Claims	33-34 and 36-37	YES
	Claims	1-4, 32, 35 and 40	NO
Industrial Applicability (IA)	Claims	1-4, 32-37 and 40	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-4, 32-37 and 40 meet the criteria set out in PCT Article 33(4), because drought-resistant plants, and methods of making them, have industrial applicability.

Claims 33-34 and 36-37 meet the criteria set out in PCT Articles 33(2)-(3), because the prior art does not teach or fairly suggest using PEG for selection of plants transformed with trehalose-6-phsophate synthase.

Claims 1-4, 32 and 40 lack novelty under PCT Article 33(2) as being anticipated by NOVARTIS (WO 99/46370).
NOVARTIS teach plastid transformation vectors encoding E. coli trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase and methods of using them to confer drought resistance to plants (pg 60-72; pg 32, lines 21-23; pg 78, lines 1-8).

Claims 1-4, 32, 35 and 40 lack an inventive step under PCT Article 33(3) as being obvious over NOVARTIS in view of Londesborough et al et al (1998, US Patent 5,792,921).
NOVARTIS et al teach a method of producing drought-resistant plants, as discussed above, but do not teach the use of a yeast gene.
Londesborough et al teach yeast genes encoding trehalose synthase and trehalose-6-phosphate phosphatase (column 26, line 26, to column 28, line 45) and plants transformed with them (column 47, line 55, to column 52, line 22).
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to produce dought resistant plants by transformation of plastids with trehalose synthase or trehalose-6-phosphate phosphatase genes as taught by NOVARTIS, and to modify that to use yeast genes as described in Londesborough et al. One of ordinary skill in the art would have been motivated to do so because the source of the genes is an obvious design choice.

Claims 1-4, 32 and 40 lack novelty under PCT Article 33(2) as being anticipated by Adams et al (1998, US Patent 5,780,709).
(Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C12N 5/10, 15/82, 5/04; A01H 4/00 and US Cl.: 800/278, 284, 288, 289; 435/320.1, 468, 410, 419, 430, 431

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
page(s) 1-21, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
page(s) 22-24, as originally filed.
page(s) NONE, as amended under Article 19.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the drawings,
page(s) 1-3 and 7, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
4-7 and 9, filed with the letter of 15 January, 2002

This report has been drawn on the basis of the sequence listing part of the description:
page(s) NONE, as originally filed.
pages(s) NONE, filed with the demand.
and additional amendments:
NONE

5. (Some) amendments are considered to go beyond the disclosure as filed:
The amendment of the description/claim filed 15 January, 2002 is objected to under PCT Article 34(2)(b) because it adds matter into the application that goes beyond the disclosure as originally filed. The added matter that is new is as follows: "antibiotic-free selectable marker sequence" in claim 1 and new claim 45; "transcriptionally active spacer sequence" in claims 2, 33, 36, 42-44 and 48-49.
Thus, the amended claims have not been entered.

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
Adams et al teach a method of producing drought-resistant plants whose plastids express mannitol-1-phosphate dehydrogenase, which produces the osmoprotectant mannitol (claim 7).

---------- NEW CITATIONS ----------
NONE

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: GUY T. DONATIELLO
SCHNADER HARRISON SEGAL & LEWIS LLP
1600 MARKET STREET, SUITE 3600
PHILADELPHIA, PA 19103-7286

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	18 JUN 2002
Applicant's or agent's file reference 1466-PCT-00	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US01/06271	28 FEBRUARY 2001	29 FEBRUARY 2000

Applicant
AUBURN UNIVERSITY

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ANNE R. KUBELIK
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)★

09/807836

PCT/US01/06271

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 19 November 2001 (19.11.01)	
International application No. PCT/US01/06271	**Applicant's or agent's file reference** 1466-PCT-00
International filing date (day/month/year) 28 February 2001 (28.02.01)	**Priority date** (day/month/year) 29 February 2000 (29.02.00)
Applicant DANIELL, Henry et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

21 September 2001 (21.09.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switz rland**	Authorized officer Sean Taylor
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

US0106271